INTRODUCTION

Presence of dual and triple viral infections has been reported from various parts of the world. Since hepatitis B, C and D share the same mode of transmission hepatitis B virus (HBV) combined infection with HCV and HDV is a fairly frequent occurrence among intravenous drug users and subjects with a high risk of parenteral infection, particularly in areas where all these viruses are endemic. It is generally considered a condition favouring the progression of liver cirrhosis and represents one of the most important risk factors for the development of hepatocellular carcinoma. Hepatitis delta virus (HDV) is a RNA viral agent that obliges hepatitis B virus for packaging and transmission. HBV provides the envelope (HBsAg) proteins for the assembly, release, and spread of virions containing the HDV RNA genome. By itself, it is unable to replicate, initiate or infect other cells completely. Compared to HDV there is confusing information about HBV and HCV behaviour during dual infection. Several reports suggest that HBV and HCV interact in the case of coinfection. In particular, in vitro studies indicate that HCV is capable of suppressing HBV activity and this inhibitory effect is essentially mediated by the HCV core protein. Around the world, the prevalence of anti-HDV has been described on the basis of interaction between the HDV and dominant HBV genotypes in specific geographic areas. However, it is not known whether such an interaction holds true in Pakistan where the most prevalent genotype is D coexisting with A and a combination of A/D.
In Pakistan, hepatitis B infection (HBV) is hyper endemic, but the frequency of co/super infection with HDV or HCV and its correlation with HBV genotypes is generally not known. There has been no report on the frequency of dual and triple infection in different categories of HBV infections and its relationship to HBV genotypes. The objective of the present study was to determine the frequency of the super infection of hepatitis C and D in patients with hepatitis B related complex liver disorders and to find out the distribution of HBV genotypes in these patients.

**METHODOLOGY**

Patients registered at the Pakistan Medical and Research Council (PMRC), Gastroenterology Unit of Jinnah Postgraduate Medical Centre (JPMC), Karachi were included. The previously diagnosed HBV positive, 180 registered patients, irrespective of age or gender, reporting for a follow-up at the Outpatient Department, were investigated for their current status and co/super infection with HDV or HCV during July 2006 and June 2007. Out of 180 patients, who were selected for study, 51 were excluded due to their incomplete test profile and follow-up.

Serum samples were collected from the study patients when they came for the follow-up. Before taking the blood sample, a written consent and in case of under 18 years of age, a parental consent was obtained. The study was approved by the Ziauddin University Ethical Review Committee. Ten ml of blood was drawn from all patients and serum was separated. All sera were stored in aliquots of 200µl each at -70°C. Clinical conditions for all patients were investigated through laboratory test results including liver function tests, coagulation profile, findings at abdominal ultrasonography, upper gastrointestinal endoscopy and liver biopsy. Liver cirrhosis and hepatocellular carcinoma (HCC) were diagnosed either on the basis of histology or on a combination of radiological, endoscopic and laboratory data of patients with HCC. The hepatitis B serology tests used were the HBe, HBe and HBs antibody tests and the microparticle enzyme immunoassay HBe/HBs antigen tests and hepatitis delta virus antibody tests (Abbott Laboratories, North Chicago, IL, USA). All tests were performed on the IMx or AxSYM system in accordance with the manufacturer’s specifications. Antibodies against hepatitis C virus were done through ortho-clinical diagnostic kit by Johnson and Johnson.

DNA extraction and amplification by PCR was done to study the HBV genotype. DNA was extracted from 200 μl of serum using a DNA extraction kit (QIA AMP DNA mini kit 250 reactions Cat #51306). Fifty micro litre of extracted DNA was concentrated by using DNA speed vac. Ten microlitre of DNA was used for PCR. The HBV genome was amplified by a modified version of nested PCR developed by Naito et al., as described previously. Each PCR reaction consisted of reaction buffer, 0.2 mmol/L deoxyribonucleotides, 1.5 mmol/L MgCl₂, 50 ng of each primer, and 2 U Taq polymerase in a final volume of 50 μL. All standard precautions were taken during the study to prevent cross contamination between PCR samples. Some of the samples were checked twice or thrice for confirmation.

For statistical evaluation frequency and percentages were calculated from categorical variables like chronic liver disease (CLD) cirrhosis, HCC and carriers. Mean values with standard deviation were calculated for continuous variables. SPSS version 13 was applied for statistical analysis.

**RESULTS**

Among the 129 patients with complete test profile finally selected for statistical evaluation, 108 (84%) were males and 21 (16%) were females with male to female ratio of 5:1. The ages of the subjects ranged from 6 - 68 years (mean=31.5 ±12.39 years).

There were 70 (54.2%) chronic liver disease (non-cirrhosis) patients, 38 (29.4%) were carriers, 12 (9.3%) were cirrhotics and 9 (6.9%) were HCC patients. Among the 129 patients, 58.9% (n=76) were not coinfected, though nine had developed HCC. Patients positive for double-active infection were 45 (34.9%) for HBV/HDV and 4 (3.1%) for HBV/HCV. Triple active infection with HBV/HDV/HCV was present in only 4 (3.1%) patients (Table I).

<table>
<thead>
<tr>
<th>Infection</th>
<th>CLD (non-cirrhosis)</th>
<th>Carrier</th>
<th>Cirrhosis</th>
<th>HCC</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV(alone)</td>
<td>30 (42.9%)</td>
<td>35 (92%)</td>
<td>2 (5.0%)</td>
<td>8</td>
<td>76</td>
</tr>
<tr>
<td>HBV/HDV</td>
<td>35 (50.0%)</td>
<td>3 (8%)</td>
<td>7 (58.3%)</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>HBV/HCV</td>
<td>1 (1.4%)</td>
<td>0</td>
<td>2 (16.7%)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>HBV/HDV/HCV</td>
<td>4 (5.7%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Infection</th>
<th>CLD (non-cirrhosis)</th>
<th>Carrier</th>
<th>Cirrhosis</th>
<th>HCC</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV(alone)</td>
<td>30 (42.9%)</td>
<td>35 (92%)</td>
<td>2 (5.0%)</td>
<td>8</td>
<td>76</td>
</tr>
<tr>
<td>HBV/HDV</td>
<td>35 (50.0%)</td>
<td>3 (8%)</td>
<td>7 (58.3%)</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>HBV/HCV</td>
<td>1 (1.4%)</td>
<td>0</td>
<td>2 (16.7%)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>HBV/HDV/HCV</td>
<td>4 (5.7%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

**HBV- hepatitis B virus, HCV- hepatitis C virus, HDV- hepatitis delta virus co-infection with HCV was detected in 8 patients; 4 as dual infection and 4 as triple infection. Super infection with HDV was present in 45 (34.9%) patients. Viral markers of hepatitis B replication, HBV DNA, was present in all of the cases. Among the 70 patients with non-cirrhosis, 50% were coinfected with HDV, 1.4% with HCV and 5.7% with triple infection. Cirrhosis had developed in 12 (9.3%) patients, out of which 25% had been infected by HBV alone whereas, 58.3% had coinfection with HDV and 16.6% with HCV. Among the 38 asymptomatic carriers, only 8% were coinfected with HDV whereas the rest (92%) were not coinfected. In HCC patients, 89% were not coinfected and had HBV alone. Anti-HDV was positive in 50% (35/70) CLD patients (33 males and 5 females), 58% (7/12) cirrhotic patients and 8% (3/38) HBV carriers but
none of the patients who had developed HCC were positive for anti-HDV. Regarding co-infection with HCV, 4/70 CLD, 2 cirrhotics and one HCC were infected. All carriers were HBeAg negative.

The genotype distribution of HBV was observed as D in 98 (76%) patients, A in 24 (18.6%), and AD in 7 (5.4%). No case of genotype B, C, E or F was found. Accordingly, genotype D strains were the predominant strains among all categories (Table II).

Regarding the genotypic distribution amongst the patients infected with both HBV/HDV, genotype D was present in 26 patients and A in 3. In patients with HBV/HCV, D was present in 2, A in one and AD in one. In patients with triple infection, HBV/HCV/HDV, genotype D was present in 3 and A in one. Thus D was found to be the dominant HBV genotype in all dual and triple infections.

### DISCUSSION

Despite widespread prevalence of hepatitis B in the Asian population, the data regarding dual or triple infection with HCV and HDV is scarce compared to HIV. In this study, 34.9% of HBV patients who came for follow-up had evidence of infection with HDV and 3.1% of HCV. Presence of triple infection with all three viruses was confirmed in only 3.1% of patients compared to that reported by other studies around the world which show a high prevalence of infection. The assessment of the level of association of dual and triple infection with different categories of HBV infections revealed that patients suffering from chronic liver disease and cirrhosis (50% and 58.2% respectively) were more likely to have dual infection with HDV compared to carriers and HCC patients.

Generally, chronic HBV infection is considered as a situation suggested to be tested for HDV infection, especially if there is acute worsening of the liver condition. Drug abusers who share contaminated needles to inject themselves with illicit drugs are likely to become infected by HDV as well. Chronic HBV carriers who acquire HDV superinfection usually develop chronic HDV infection. Cirrhosis and hepatocellular carcinoma (HCC) are two major long-term complications of chronic HBV infection which developed in 21 patients out of 129. The effect of hepatitis delta virus (HDV) infection on the clinical course of cirrhosis is poorly defined, yet it has been reported that HDV infection increases the risk for mortality two-fold in patients with cirrhosis type B. The development of cirrhosis occurs more frequently in patients with episodes of decompensation and with repeated severe acute exacerbations. However, progression to cirrhosis can be relatively silent and can occur even in children. Although cirrhosis develops during the process of HBeAg seroconversion, 68% of the complications of cirrhosis and of hepatocellular carcinoma occur after HBeAg seroconversion. These complications may still occur even after HBSAg seroclearance. Basically, the virus produces many of the same diseases as the hepatitis B virus; however, these problems such as cirrhosis are much more deadly and frequent. Out of the 12 patients (9.3%) who had developed cirrhosis in this study, 7 were HDV positive and 2 HCV, whereas, only 3 patients developed cirrhosis due to HBV alone.

Similarly, regarding HCC, it has been reported that HDV superinfection developed HCC at an earlier age than HBsAg carriers without HDV infection. Hepatitis D virus appears to represent a promotion factor for HCC in subjects with an oncogenic risk induced by HBV, increasing the risk for HCC three-fold. In comparison to these reports, the 9 HCC patients in this study, ages 35-55 years, did not have dual or triple infection, except for one who was HCV positive. It seems HBV alone was responsible for this complication in 8 patients. An association between HDV infection, with a higher rate of HCC, was also found in a Japanese study; however, they could not conclusively confirm this association. Nevertheless, there seems to be a fair amount of evidence that HDV infection can lead to HCC. A classic form of chronic HBV/HCV infection, identified by the positivity of the HBV surface antigen (HBsAg) and the antibody to HCV (anti-HCV) was observed in 4 patients. It is generally considered a condition favouring the progression of liver fibrosis and the establishment of cirrhosis. It also represents one of the most important factors for the development of hepatocellular carcinoma which was observed in only one female with HCC in this study.

Asymptomatic carriers in this study were 38 (29.4%), out of which only 3 (8%) were coinfected with HDV and 35 (92%) were not coinfected. Super infection with HDV in HBV carriers in Western countries has been reported to aggravate the clinical course of chronic hepatitis B but

### Table II: Distribution of HBV genotypes in different categories of patients.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CLD (non cirrhosis)</th>
<th>Carrier</th>
<th>Cirrhosis</th>
<th>HCC</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males (%)</td>
<td>Females (%)</td>
<td>Males (%)</td>
<td>Females (%)</td>
<td>Males (%)</td>
</tr>
<tr>
<td>D</td>
<td>59 (84)</td>
<td>11 (16)</td>
<td>31 (89)</td>
<td>7 (18.4)</td>
<td>8 (80)</td>
</tr>
<tr>
<td>A</td>
<td>9 (15)</td>
<td>3 (27)</td>
<td>6 (19)</td>
<td>-</td>
<td>2 (20)</td>
</tr>
<tr>
<td>AD</td>
<td>4 (7)</td>
<td>-</td>
<td>4 (13)</td>
<td>2 (28)</td>
<td>-</td>
</tr>
</tbody>
</table>

the infection does not seem to affect the liver disease in Asian HBsAg carriers.\(^{17,18}\) The less aggressive course of HDV infection in Asia than in Western countries may be explained by the distribution of different genotypes of HDV in specific geographic areas which were not checked in this study due to study limitations. The predominant genotype of hepatitis B in Pakistan is genotype D.\(^{9,10}\) The HDV genotype reported from Pakistan is I; the most prevalent genotype worldwide which is associated with a broad spectrum of pathogenesis.\(^{19}\) A correlation of HBV genotype D with HDV genotype I has been reported from other countries such as Italy, Turkey and Egypt.\(^{20-22}\) In studies from other developing countries such as Taiwan and Vietnam where HBV infection is hyperendemic,\(^{23,24}\) HDV infection has also been reported to be generally infrequent in asymptomatic HBsAg carriers, whereas, it is extremely high in intravenous drug users.\(^{17}\) They also reported it to be infrequent in CLD which is in contrast to this study.

Different genotypes of the hepatitis viruses may influence the clinical outcome of the disease. The genotype distribution of HBV in this study showed predominance of genotype D (76%) in all categories, A being the second most (18.6%), and a combination of both the least as AD (5.4%). The secretion of HDV generally correlates with HBsAg levels, but not with HBV genotypes or HBV DNA levels.\(^{25}\) These results of HBV genotype distribution are comparable to a previously reported study in which the genotype D was 70.9%, A, 20.2% and AD 9.1%.\(^{9}\) Genotype A was dominant in HCC patients in that study, whereas, in the present study genotype D was dominant in 8/9 HCC patients and only one had A. The distribution of genotypes vary according to geographical regions and so does its virulence. The HBV isolates coinfected with HDV found on Miyako Island were of genotype B.\(^{23}\) In Turkey, patients with chronic viral hepatitis showed very little genotypic heterogeneity. Subtype ayw and the genotype D of HBV DNA and type I of HDV RNA represented almost 100% of related infections. There were a total of 8 cases of HCV, 4 in dual infection and 4 in triple infection. The genotypic correlation of HBV and HCV is not known. There are 6 known genotypes and more than 50 subtypes of hepatitis C.\(^{26}\) HCV genotypes 1, 2, and 3 appear to have a worldwide distribution and their relative prevalence varies from one geographic area to another. The most common HCV genotype in Pakistan is type 3a. More than 70% of the cases are acquired in hospitals through the reuse of needles/syringes or major/minor surgery which is a common practice in this country.\(^{27}\)

### CONCLUSION

The frequency of super infection of hepatitis C and D was found to be highest in HBV cirrhosis patients compared to patients having chronic liver disease (non-cirrhosis) and carriers. Genotype D of the hepatitis B virus was found to be dominant in all hepatitis B related complex liver disorders. The delta virus may be considered to be more infectious in causing super infection in HBV patients compared to the hepatitis C virus. These findings prompt further investigation into the relationship between HDV genetic structures and their function and pathogenesis.

**Acknowledgement:** This study was supported by a grant from the Pakistan Medical Research Council (PMRRC). The authors are thankful to Syed Ejaz Alam for assistance in statistical analysis.

### REFERENCES

13. Lu SN, Chen TM, Lee CM, Wang JH, Tung HD, Wu JC. Molecular epidemiological and clinical aspects of hepatitis D in


27. Idrees M, Riazuddin S. Frequency distribution of hepatitis C virus genotypes in different geographical regions of Pakistan and their possible routes of transmission. BMC Infect Dis 2008; 8:69.